

# Physico-chemical characterization of protein-associated polysaccharides extracted from sugar beet pulp<sup>☆</sup>

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## ABSTRACT

We have solubilized and separated polysaccharides from sugar beet pulp (SBP) into three fractions with steam assisted flash extraction (SAFE). For pectin, recovery ranged from 8 to 14%, degree of methy-esterification 66–73%, crude protein 1.3–1.7%,  $M_w$  262–318 kDa,  $\eta_w$  0.22–0.23 dL/g,  $Rg_z$  36–39 nm and  $Rh_z$  41–42 nm. For alkaline soluble polysaccharides, (ASP I) recovery ranged from 4.0 to 6.5%, crude protein 1.2–4.8%, weight average molar mass ( $M_w$ ) 66–68 kDa, weight average intrinsic viscosity ( $\eta_w$ ) 0.27–0.30 dL/g, z-average radius of gyration ( $Rg_z$ ) 25–29 nm and z-average hydrated radius ( $Rh_z$ ) 10–11 nm. ASP II recovery ranged from 2.0 to 8.6%, crude protein 1.2–4.8%,  $M_w$  299–339 kDa,  $\eta_w$  0.22–0.33 dL/g,  $Rg_z$  33–34 nm and  $Rh_z$  30–34 nm. Recovery of the residue mainly cellulose, ranged from 20.3 to 22.3%. The cellulose in this fraction was converted to carboxymethyl cellulose (CMC). The CMC fraction contained 0.33–0.43 crude protein and had an  $M_w$  ranging from 127 to 263 kDa,  $\eta_w$  3.6–8.0 dL/g,  $Rg_z$  35–45 nm and  $Rh_z$  27–40 nm.

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## 1. Introduction

The extraction of sugar from sugar beets in the U.S. produces about two million tons of SBP each year (Schwartz, 2006). On a dry weight basis, SBP is a rich source of carbohydrates in that it contains 67–80% of potentially high valued plant cell wall polysaccharides (Oosterveld, Beldman, Schols, & Voragen, 1996; Sun & Hughes, 1999). About 19–25% of these polysaccharides are pectin, 21–30% pectin-associated arabinan and/or galactans, and 22–24% cellulose (Buchholt, Christensen, Fallesen, Ralet, & Thibault, 2004; Oosterveld et al., 1996; Sun & Hughes, 1999). Isolation and characterization of the functional properties of these polysaccharides could increase the value of SBP over its value as an animal feed which is now its primary use.

Previously, we sequentially extracted and characterized, pectin, alkaline soluble polysaccharides (ASP) by microwave-assisted flash extraction, (MAFE). Residual cellulose was converted to CMC from SBP (Fishman, Chau, Cooke, & Hotchkiss, 2008; Fishman, Chau, Cooke, Yadav, & Hotchkiss, 2009; Fishman et al., 2011). In

the case of pectin we found that we could extract high molar mass, moderate viscosity pectin in minutes rather than hours as required by conventional heating methods. MAFE allowed us to obtain ASP in minutes with an  $M_w$  of about 100 kDa. Also, we were able to convert residual cellulose to CMC with  $M_w$  as high as 160 kDa. In another study, we obtained pectin in laboratory quantities from orange albedo by steam-assisted flash extraction (SAFE) which was comparable in physical properties to pectin from the same source obtained by MAFE (Fishman, Walker, Chau, & Hotchkiss, 2003). In this work we obtained pectin and ASP prior to the conversion of the residue to the sodium salt of carboxymethyl cellulose (SCMC) by a scaled up SAFE and characterized them by HPSEC with on-line multi-angle light scattering (MALLS), viscosity (DP), refractive index (RI) and ultra violet (UV) detection. Leroux, Langendorff, Schick, Vaishnav, and Mazoyer (2003) reported that sugar beet pectin reduced the interfacial tension between an oil and water phase which they attributed to the presence of acetyl groups and hydrophobic proteins in SBP preparations. Funami et al. (2007) concluded that the proteinaceous moiety in SBP increases the surface activity of SBP and it also increases the accessibility of SBP to emulsion droplets. Williams et al. (2005) fractionated SBP using hydrophobic affinity chromatography and reported that there were significantly different proportions of protein and ferulic acid in the various fractions, but that there was no simple relationship between protein or ferulic acid content and the extent to which these fractions act as emulsifiers.

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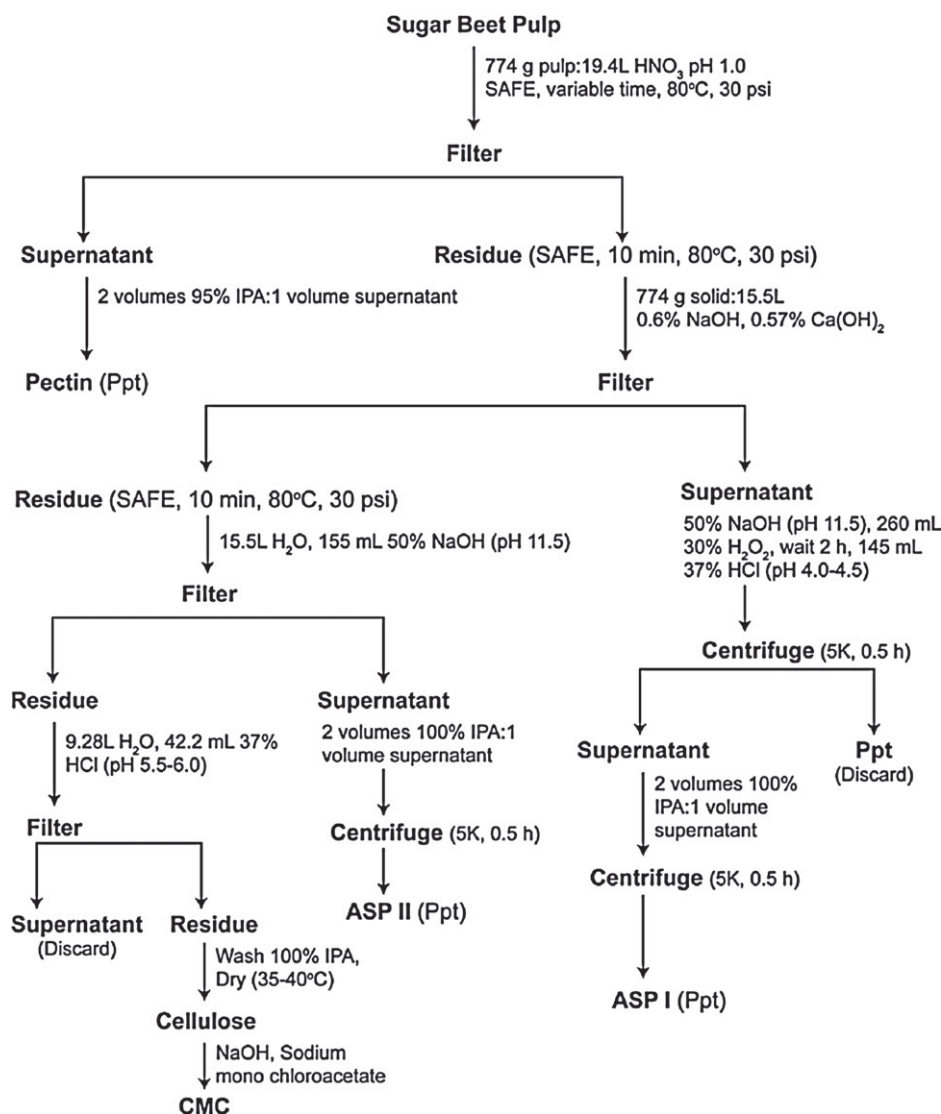


Fig. 1. Flow diagram for the SAFE extraction of polysaccharides from sugar beet pulp.

Recently, Nunez, Fishman, Fortis, Cooke, and Hotchkiss (2009), analyzed the peptides in protein associated with SBP by matrix-assisted laser desorption ionization with tandem time-of-flight mass spectrometry (MALDI-TOF/TOF-MS). The tryptic peptide sequences obtained were found to be highly consistent with those from extensin protein. Also Nunez et al. (2009) established from atomic force micrographs that the number of rod-like elements present decreased after protease treatment. Furthermore, Fishman et al. (2009) found that protein also was present in ASP extracted from sugar beet pulp. Preliminary experiments appeared to indicate that ASP also may act as an emulsifier in food systems.

The objectives of this research were twofold. To determine if the SAFE process could be scaled up to produce pilot plant quantities of valuable polysaccharides that were extracted from sugar beet pulp and to determine the distribution of protein moieties in these extracted polysaccharides.

## 2. Materials and methods

### 2.1. Materials

Partially dewatered sugar beet pulp with sugar removed was donated by American Crystal Sugar Co., Moorhead, MN. The pulp

was shipped frozen and stored at  $-20^{\circ}\text{C}$  until prepared for extraction. The frozen pulp was ground with a Comitrol 1700 fitted with a micro cut cutting head (Urschel Laboratories, Valparaiso, IN). In order to keep it cool while grinding, dry ice was added with the pulp. Commercial sugar beet pectin was obtained from CP Kelco (San Diego, CA) and bovine serum albumin (ca. 98% monomer) from Sigma–Aldrich Co. (St. Louis, MO). Both materials were used without further purification. Sugar beet pectin and alkaline soluble polysaccharides were prepared by SAFE as described previously (Fishman et al., 2008, 2009).

### 2.2. Steam assisted flash extraction (SAFE)

The flow diagram for the extraction of the various polysaccharides is illustrated in Fig. 1. Briefly, sugar beet pulp and the extraction solution were mixed, stirred overnight in a cold room and added to a modified 60 L fermentor (Stainless and Steel Products, St. Paul, Minnesota). After addition, the slurried pulp was continuously mixed using a circulation pump (Model S100-33 Price Pump Co., East Sonoma, CA) (Fig. 2). The circulation pump was expressly incorporated into the fermentor to thoroughly mix the slurry and evenly distribute the clean steam used for heating. Also, the fermentor was fitted with  $\frac{1}{2}$  in. national pipe threads (NPT)

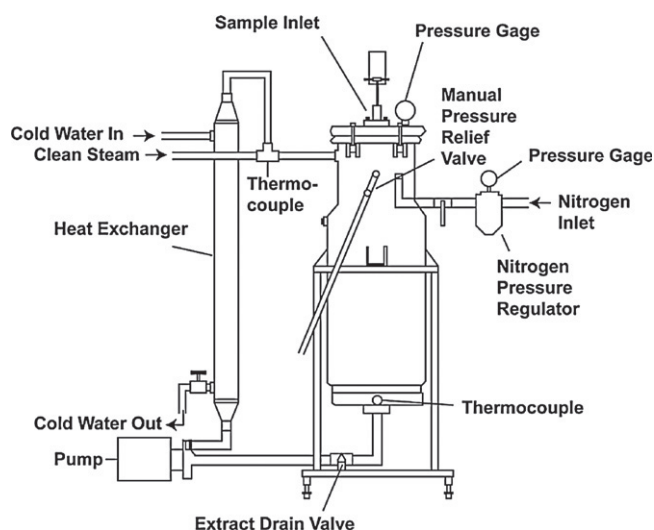


Fig. 2. Schematic of SAFE extraction vessel.

pipings to supply deionized clean steam and nitrogen to maintain the desired temperature and pressure. Furthermore, thermocouples and pressure gauges were incorporated into the system to monitor and aid in controlling the temperature and pressure within the fermentor. The slurry was heated with 17.2 psi steam, until the desired temperature was reached. While the slurry was being heated, the system was periodically vented to keep it at atmospheric pressure. When the desired temperature was achieved within the fermentor, nitrogen, an inert gas, was streamed into the fermentor to maintain the pressure at 30 psi. These conditions were maintained for variable times which depended on the specific experiment being conducted. Once the desired heating time was reached, the slurry was cooled to room temperature by circulating it through a shell and bundled tubes heat exchanger (Type MA11T-1-E-SS, Ametek, Grand Prairie, TX) until it was brought to

room temperature. This is achieved by allowing the hot slurry to flow through the heat exchanger tubes while the cooling water runs over the outside of the tubes thereby allowing the heat to transfer from the hot slurry to the cold water. The slurry was then drained from the fermentor vessel and pectin was isolated from the supernatant by filtration or centrifugation (Type AS26-SAN-485146, Sharples Philadelphia, PA). After ASP I or ASP II was isolated from the supernatant (see Fig. 1) they were filtered and/or centrifuged. After filtration or centrifugation one volume of supernatant was mixed with two volumes of 95% isopropyl alcohol (IPA) and was left to sit for an hour to allow the polysaccharide to precipitate. The precipitated polysaccharide was separated from the liquid layer by filtering through cheese cloth.

The polysaccharide was then vacuum dried overnight (New York Engineering Co., Yonkers, NY) and stored under vacuum until analyzed.

The extracted residue was washed with 100% IPA, dried at 35–40 °C, and stored in vials until carboxymethylated as described in the next section.

### 2.3. Chemical composition

Methods for the determination of percentage recoveries of polysaccharides (Table 1), compositional analysis of polysaccharides (Table 2), monosaccharide analysis (Table 3), percentage nitrogen and crude protein present in isolated polysaccharides (Table 4) have been described previously (Fishman et al., 2009).

### 2.4. Carboxymethylation (CM) of the SBP residue after acid/alkali extraction and determination of degree of substitution (DS)

CM of the SBP residue and determination of the DS have been described previously (Fishman et al., 2011). The previously extracted residue was carboxymethylated according to the method of Heinze and Pfeiffer (1999). One gram of SBP residue was slurried in 30 mL of isopropanol. An aqueous solution (2.66 mL) containing 15% NaOH was added to the mixture and stirred for 1 h.

Table 1

Mean percent recoveries of polysaccharides in sugar beet pulp.

Sample	Pectin <sup>a</sup>	ASP I <sup>b</sup>	ASP II <sup>b</sup>	Cellulose	Total
1	7.7 (2) <sup>c</sup>	6.5 (2)	8.6 (1)	22.3 (1)	45.1 (6)
2	13.6 (4)	4	2	20.6	40.2

<sup>a</sup> Pectin extraction conditions; sample 1, 6/80/30 (min/°C/psi); sample 2, 8/80/30 (min/°C/psi).

<sup>b</sup> ASP I and II extractions; samples 1 and 2, 10/100/30 (min/°C/psi).

<sup>c</sup> Standard deviation of triplicate analysis.

Table 2

Compositional analysis in mole percent of polysaccharides in sugar beet pulp.

Sample	Polysaccharide	GA <sup>b</sup>	DE <sup>b</sup>	DA <sup>b</sup>	NS <sup>b</sup>
1 6/80/30 <sup>a</sup>	Pectin	65.8 (0.8) <sup>c</sup>	66.2 (4)	23.4 (2)	40.3 (2)
2 8/80/30 <sup>a</sup>	Pectin	64.6 (4)	73.2 (6)	28.7 (2)	41.6 (1)
1 10/100/30 <sup>b</sup>	ASP I	26.8 (3)	ND <sup>d</sup>	ND	51.8 (1)
2 10/100/30	ASP I	30.9 (2)	ND	ND	54.9 (1)
1 10/100/30	ASP II	37.5 (2)	ND	ND	38.0 (1)
2 10/100/30	ASP II	67.7 (4)	ND	ND	38.0 (1)
1 15–5 <sup>c</sup>	CMC (S) <sup>f</sup>	11.7 (2)	ND	ND	23.5 (1)
2 15–5 <sup>c</sup>	CMC (S)	13.1 (2)	ND	ND	23.7 (2)
1 15–5	CMC (S+I) <sup>g</sup>	7.3 (1)	ND	ND	42.7 (1)
2 15–5	CMC (S+I)	9.4 (2)	ND	ND	26.1 (2)

<sup>a</sup> Pectin extraction conditions (min/°C/psi).

<sup>b</sup> ASP extraction conditions (min/°C/psi).

<sup>c</sup> Reaction conditions for carboxymethylation of cellulose, 15% NaOH, 5 h heating.

<sup>d</sup> Not detected.

<sup>e</sup> Standard deviation of triplicate analysis.

<sup>f</sup> Soluble fraction.

<sup>g</sup> Total soluble and insoluble fraction.

<sup>h</sup> GA, galacturonate; DE, degree of methyl esterification; DA, degree of acetylation; NS, neutral sugars.

**Table 3**

Relative mole percent of monosaccharides in sugar beet pulp polysaccharides.

	Fuc <sup>f</sup>	Rha <sup>f</sup>	Ara <sup>f</sup>	Gal <sup>f</sup>	Glc <sup>f</sup>	Xyl <sup>f</sup>	Gal A <sup>f</sup>	Glc A <sup>f</sup>
1 Pectin <sup>a</sup>	0.2	3.4	22.5	7.3	2.54	0.3	62.5	1.3
2 Pectin <sup>a</sup>	0.2	5.9	14.1	11.4	3.74	0.4	62.7	1.5
1 ASP I <sup>b</sup>	0.1	18.7	32.9	28.6	0.4	0.3	16.4	2.7
2 ASP I <sup>b</sup>	0.14	21.0	23.9	36.2	0.6	0.5	14.8	2.8
1 ASP II <sup>b</sup>	0.3	14.3	25.7	24.2	1.2	1.0	29.6	3.7
2 ASP II <sup>b</sup>	0.23	11.1	15.3	16.8	0.8	0.7	53.4	1.8
1 CMC (S) <sup>c,d</sup>	0.2	0.2	0.3	0.3	54.4	4.3	1.6	38.7
2 CMC (S) <sup>c,d</sup>	0.1	0.3	0.2	0.1	52.1	3.8	1.0	42.3
1 CMC (S+I) <sup>c,e</sup>	0.2	0.3	0.9	0.5	59.2	6.0	1.8	31.0
2 CMC (S+I) <sup>c,e</sup>	0.1	0.2	0.2	0.1	48.2	2.7	1.5	47.0

<sup>a</sup> Pectin extraction conditions; sample 1, 6/80/30; sample 2, 8/80/30 (min/°C/psi).<sup>b</sup> ASP I and II extractions; samples 1 and 2, 10/100/30 (min/°C/psi).<sup>c</sup> Obtained after pectin and ASP extracted by SAFE followed by pulp treatment, 15% NaOH, heated for 5 h.<sup>d</sup> Soluble in 0.05 M NaNO<sub>3</sub> and 0.01% NaN<sub>3</sub>.<sup>e</sup> Total soluble and insoluble in 0.05 M NaNO<sub>3</sub> and 0.01% NaN<sub>3</sub>.<sup>f</sup> Fuc, fucose; Rha, rhamnose; Ara, arabinose; Gal, galactose; Glc, glucose; Xyl, xylose; Gal A, galacturonic acid; Glc A, glucuronic acid.

Then 1.2 g of sodium monochloroacetate was added to the mixture and heated for 5 h at 55 °C. The mixture was filtered and the resultant filtrate was suspended in 60 mL of aqueous methanol (80%, v/v), neutralized with acetic acid and filtered again. The solid product was washed three times with 60 mL of 80% aqueous ethanol and once with 60 mL of anhydrous ethanol followed by drying under vacuum at 60 °C and stored in vials for future use.

FT-IR spectra of CMC were used to determine its degree of substitution (DS) according to the method and parameters of Pushpamalar, Langford, Ahmad, and Lim (2006). FT-IR spectra were collected with a Thermo Electron Nexus 670 FT-IR spectrometer (Madison, WI). Fifty milligram of the CMC was dissolved in 5 mL of water with stirring overnight. The resulting solution was centrifuged for 30 min at 50,000 × g in a Beckman Coulter Optima TLX Ultracentrifuge (Fulerton, CA). Ten drops of the supernatant were placed on a CaF<sub>2</sub> plate, allowed to air dry overnight and then vacuum dried for 20 min. The films were scanned from 1200 to 3700 cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup>. All spectra were obtained by averaging 64 scans. The degree of substitution was calculated from the methyl (CH) stretching absorption peak at absorbance 2920 cm<sup>-1</sup> and the carboxyl absorbance at 1605 cm<sup>-1</sup> as described by Pushpamalar et al. (2006).

### 2.5. High performance size exclusion chromatography (HPSEC)

Dried pectin (1 mg/mL) was dissolved in mobile phase (0.05 M NaNO<sub>3</sub> and 0.01% NaN<sub>3</sub>), centrifuged in a Sorval RC-5B centrifuge (DuPont Co., Wilmington, DE) at 50,000 × g, 35 °C and filtered through a 0.22 μm or 0.45 μm Millex HV filter (Millipore Corp., Bedford, MA). The procedure for the solution of dried ASP and CMC was identical to that for pectin except that prior to centrifugation

these were dialyzed for two days against mobile phase with two changes of dialysate. The flow rate for the solvent delivery system, model HLC-8320GPC EcoSEC series with degasser, auto sampler and pump, UV-8320 and RI detector (Tosoh Bioscience, Tokyo, Japan) was 0.7 mL/min. The injection volume was 200 μL. Samples were run in triplicate. The column set consisted of three model TSK GMPW<sub>XL</sub> size exclusion columns (Tosoh Bioscience, Tokyo, Japan) in series. The columns were in a heated column oven set at 35 °C. The chromatograph was fitted with a UV-8320 spectrophotometer (Tosoh Bioscience, Tokyo, Japan), HELEOS II multi-angle laser light scattering photometer (MALLS) (Wyatt Technology, Santa Barbara, CA) with measurement of quasi-elastic scattering (QELS) capability at 120°, model 255-V2 differential pressure viscometer (DPV) (Wyatt Technology, Santa Barbara, CA) and RI detector (Tosoh Bioscience, Tokyo, Japan). *M<sub>w</sub>* was calculated with the Zimm equation. The UV, MALLS, DPV and RI detectors were aligned with bovine serum albumin (BSA-#127k7405 Sigma-Aldrich, St. Louis, MO). As evidence that the detectors were aligned, RI gave a molar mass of 67 kDa for BSA whereas UV278 nm gave a molar mass of 66 kDa. Hirayama, Akashi, Furuya, and Fukuhara (1990) using electrospray ionization mass spectrometry found a BSA molar mass of 66.43 kDa. Sugar beet pectin was measured at 250 nm, 278 nm and 310 nm with the UV detector. The wavelengths at 250 nm and 310 nm were chosen because sugar beet pectin had UV absorbance peaks at ~250 nm, and ~310 nm, Williams et al. (2005) whereas ferulic acid had an absorption peak at ~319 nm (Sebastian, Sundaraganesan, & Manoharan, 2009). Shodex Standard Pullulan-P82, Lot#30901, molar mass 82 kDa (JM Science, Grand Island, NY) was used to calibrate the scattering intensity at the 90° angle. The *dn/dc* values of concentration measurements for sugar beet pectin, ASP I and II, and CMC were 0.132, 148 and 0.150 respectively.

**Table 4**

Percentage nitrogen and crude protein (6.25N) in polysaccharides from sugar beet pulp.

Sample	Polysac	Soluble (S) <sup>d</sup>		Insoluble (I)		S+I <sup>e</sup>	
		Nitrogen	Protein	Nitrogen	Protein	Nitrogen	Protein
1 6/80/30 <sup>a</sup>	Pectin	0.91 (.02)	5.7	0.80 (.02)	5.0	1.71 (.04)	10.7
2 8/80/30	Pectin	0.58 (.01)	3.6	0.76 (.01)	4.8	1.34 (.01)	8.4
1 10/100/30 <sup>b</sup>	ASP I	0.21 (.01)	1.3	0.98 (.01)	6.1	0.20 (.02)	1.2
2 10/100/30	ASP I	0.65 (.02)	4.1	4.33 (.08)	27	0.77 (.02)	4.79
1 10/100/30	ASP II	0.55 (.01)	3.4	0.94 (.01)	5.9	0.61 (.02)	4.11
2 10/100/30	ASP II	2.2 (.01)	13.7	1.16 (.01)	7.3	1.38 (.02)	8.6
1 15–5 <sup>c</sup>	CMC <sup>c</sup>	0.03 (.02)	0.2	0.30 (.02)	1.9	0.05 (.01)	0.33
2 15–5	CMC <sup>c</sup>	0.05 (.01)	0.3	0.22 (.01)	1.4	0.07 (.01)	0.43

<sup>a</sup> Pectin extraction conditions (min/°C/psi).<sup>b</sup> ASP extraction conditions (min/°C/psi).<sup>c</sup> Reaction conditions for carboxymethylation of cellulose, 15% NaOH, heated for 5 h.<sup>d</sup> Soluble in 0.05 M NaNO<sub>3</sub> and 0.01% NaN<sub>3</sub> after drying.<sup>e</sup> Total soluble and insoluble in 0.05 M NaNO<sub>3</sub> and 0.01% NaN<sub>3</sub>.



**Table 5**Weight average molar mass ( $M_w$ ) and weight average intrinsic viscosity ( $\eta_w$ ) for polysaccharides in sugar beet pulp.

Sample	$M_w$ (kDa)				$\eta_w$ (dL/g)			
	RI	250 nm	278 nm	310 nm	RI	250 nm	278 nm	310 nm
Com Pectin <sup>a</sup>	388 (7) <sup>f</sup>	392 (10)	378 (4)	414 (8)	2.2 (0.1)	2.2 (0.1)	2.1 (0.1)	2.1 (0.1)
MPectin <sup>b</sup>	700 (15)	639 (51)	644 (14)	588 (2)	3.4 (0.1)	3.2 (0.1)	3.2 (0.1)	3.1 (0.1)
1 SPectin <sup>c</sup>	262 (7)	265 (3)	272 (6)	271 (2)	2.3 (0.1)	2.3 (0.1)	2.3 (0.1)	2.3 (0.1)
2 SPectin <sup>c</sup>	318 (2)	317 (2)	315 (2)	310 (1)	2.2 (0.1)	2.2 (0.1)	2.2 (0.1)	0.21 (0.01)
1 ASP I <sup>d</sup>	68 (1)	68 (1)	60 (1)	53 (2)	0.27 (0.01)	0.27 (0.01)	0.27 (0.01)	0.26 (0.01)
2 ASP I	66 (1)	64 (3)	61 (3)	58 (6)	0.30 (0.01)	0.30 (0.01)	0.31 (0.01)	0.30 (0.01)
1 ASP II <sup>d</sup>	266 (2)	266 (3)	312 (2)	282 (3)	0.22 (0.01)	0.22 (0.01)	0.22 (0.01)	0.21 (0.01)
2 ASP II	339 (1)	340 (1)	372 (5)	403 (5)	0.33 (0.01)	0.29 (0.01)	0.30 (0.01)	0.29 (0.01)
1 CMC (S) <sup>e</sup>	263 (4)	ND <sup>g</sup>	ND	ND	8.0 (1)	ND	ND	ND
2 CMC (S)	127 (1)	ND	ND	ND	3.6 (0.1)	ND	ND	ND

<sup>a</sup> Commercial sugar beet pectin.<sup>b</sup> Microwave assisted extraction conditions; 3/60/30 (min/°C/psi).<sup>c</sup> Steam assisted flash extraction conditions. Sample 1, 6/80/30; sample 2, 8/80/30 (min/°C/psi). Soluble in 0.05 M NaNO<sub>3</sub> and 0.01% NaN<sub>3</sub>.<sup>d</sup> ASP I and II steam assisted flash extraction conditions; all samples 10/100/30 (min/°C/psi) extraction; samples 1 and 2, 10/100/30 (min/°C/psi).<sup>e</sup> Carboxy methyl cellulose obtained after pectin and ASP extracted by SAFE followed by pulp treatment 15% NaOH, heated for 5 h.<sup>f</sup> Standard deviation of triple analysis.<sup>g</sup> Not detected.

The extinction coefficient for each wavelength was determined from the RI concentration of each individual sample. The electronic outputs from all the scattering angles measured by the UV, MALLS, DPV and RI were sent to a directory for processing with ASTRA V 5.3.4.16 software (Wyatt Technology, Santa Barbara, CA).

### 3. Results and discussion

#### 3.1. Recovery and chemical composition of polysaccharides

In Table 1 are the percentage recoveries of polysaccharides in SBP from a triplicate set of extractions. Total recovery of all polysaccharides ranged from about 40 to 45% of starting material. About 8–14% was pectin, 6–15% was ASP and 21–22% was cellulose. By way of comparison microwave assisted flash extraction gave recoveries of about 12% for pectin, 26% for ASP and 21% for cellulose.

Tables 2 and 3 respectively, contain the compositional analysis of the polysaccharides in SBP and monosaccharide analyses. In Table 2, the relatively large amount of galacturonate (GA) in ASP I and ASP II indicates that pectin is associated with the ASP fractions.

In Table 3, the relatively large amounts of arabinose and galactose and nearly 1:1 Rha:GalA ratio in the ASP I fraction indicates the possible presence of a significant rhamnogalacturonan I (RG-I) region in the associated pectin (Morris, Ralet, Bonnin, Thibault, & Harding, 2010). The degree of carboxymethyl substitution (DS) values for samples 1 and 2 was found to be 1.1 (0.1) and 1.0 (0.1)

respectively (data not included in Table 3). Furthermore, the significant amounts of glucuronic acid in the CMC fractions are probably due to carboxymethylated glucose co-eluting with glucuronic acid (Fishman et al., 2011).

In Table 4, all but the CMC fractions contain appreciable amounts of crude protein. The presence of crude protein in the ASP I and ASP II fractions may arise partially from the hydrophobic nature of the proteins which are associated with pectin in sugar beets (Leroux et al., 2003; Nunez et al., 2009). The percentage of galacturonic acid was plotted against percentage crude protein for the pectin, ASP I and ASP II fractions of sugar beet (plot not shown). The correlation coefficient for this linear least squares plot of 0.87 indicates a high correlation between galacturonic acid and crude protein. The linear least squares slope is 5.2 and at the intercept where protein is 0%, galacturonic acid is 8.1%. One possible interpretation of this data is that about 8.1% of the galacturonic acid present is not associated with protein and that the ratio of galacturonic acid to pectin associated with protein is about 5–1. This interpretation is consistent with the HPSEC analysis which will follow.

#### 3.2. Characterization of the molecular properties of sugar beet pectin

Tables 5–7 contain the results of physical measurements on polysaccharides extracted from sugar beet pulp. Comparison revealed that the  $M_w$  of pectin (Table 5) decreased in the order,

**Table 6**z-Average radius of gyration ( $R_g$ ) and z-average hydrated radius ( $R_h$ ).

	$R_g$ (nm)				$R_h$ (nm)			
	RI	250 nm	278 nm	310 nm	RI	250 nm	278 nm	310 nm
Com Pectin <sup>a</sup>	41 (1) <sup>f</sup>	41 (1)	41 (1)	41 (1)	40 (1)	26 (1)	26 (1)	28 (1)
MPectin <sup>b</sup>	38 (1)	39 (1)	40 (1)	39 (1)	42 (1)	32 (1)	36 (1)	34 (1)
1 SPectin <sup>c</sup>	36 (1)	36 (1)	37 (1)	37 (1)	41 (1)	21 (1)	22 (1)	23 (1)
2 SPectin <sup>c</sup>	39 (1)	39 (1)	40 (2)	39 (1)	42 (1)	42 (1)	23 (1)	23 (1)
1 ASP I <sup>d</sup>	29 (3)	28 (1)	19 (4)	19 (4)	10 (1)	9.6 (0.4)	7.7 (0.2)	8.1 (0.4)
2 ASP I <sup>d</sup>	25 (3)	25 (3)	21 (6)	21 (2)	11 (1)	11 (1)	10 (1)	8.2 (0.1)
1 ASP II <sup>d</sup>	34 (1)	36 (1)	36 (1)	36 (1)	30 (1)	4.1 (0.2)	17 (1)	16 (1)
2 ASP II <sup>d</sup>	33 (1)	33 (1)	33 (1)	36 (2)	34 (1)	36 (1)	36 (1)	9.4 (0.2)
1 CMC (S) <sup>e</sup>	45 (1)	ND <sup>g</sup>	ND	ND	40 (1)	ND	ND	ND
2 CMC (S) <sup>e</sup>	35 (1)	ND <sup>g</sup>	ND	ND	27 (1)	ND	ND	ND

<sup>a</sup> Commercial sugar beet pectin.<sup>b</sup> Microwave assisted extraction conditions; 3/60/30 (min/°C/psi).<sup>c</sup> Steam assisted flash extraction conditions. Sample 1, 6/80/30; sample 2, 8/80/30 (min/°C/psi). Soluble in 0.05 M NaNO<sub>3</sub> and 0.01% NaN<sub>3</sub>.<sup>d</sup> ASP I and II steam assisted flash extraction conditions; all samples 10/100/30 (min/°C/psi).<sup>e</sup> Carboxy methyl cellulose obtained after pectin and ASP were extracted by SAFE, followed by pulp treatment with 15% NaOH heated for 5 h.<sup>f</sup> Standard deviation of triple analysis.<sup>g</sup> Not detectable.

**Table 7**Ratio ( $\rho$ ) of z-average radius of gyration ( $R_{gz}$ ) over z-average hydrated radius ( $R_{hz}$ ).

	$R_{gz}/R_{hz}$			
	RI	310 nm	250 nm	278 nm
Com Pectin <sup>a</sup>	1.0	1.6	1.6	1.5
MPectin <sup>b</sup>	0.90	1.2	1.1	1.1
1 SPectin <sup>c</sup>	0.88	1.7	1.7	1.6
2 SPectin <sup>c</sup>	0.92	0.92	1.7	1.7
1 ASP I <sup>d</sup>	2.9	2.9	2.5	2.3
2 ASP I <sup>d</sup>	2.3	2.3	2.1	1.6
1 ASP II <sup>d</sup>	1.1	ND	2.1	2.3
2 ASP II <sup>d</sup>	0.97	0.92	0.92	ND
1 CMC (S) <sup>e</sup>	1.1	ND <sup>f</sup>	ND	ND
2 CMC (S) <sup>e</sup>	1.3	ND	ND	ND

<sup>a</sup> Commercial pectin.<sup>b</sup> Microwave assisted extraction conditions; 3/60/30 (min/°C/psi).<sup>c</sup> Steam assisted flash extraction conditions. Sample 1, 6/80/30; sample 2, 8/80/30 (min/°C/psi). Soluble in 0.05 M NaNO<sub>3</sub> and 0.01% NaN<sub>3</sub>.<sup>d</sup> ASP I and II steam assisted flash extraction conditions; all samples 10/100/30 (min/°C/psi).<sup>e</sup> Carboxy methyl cellulose obtained after pectin and ASP were extracted by SAFE followed by pulp treatment with 15% NaOH heating for 5 h.<sup>f</sup> Not detectable.

MAFE pectin (MPectin) > commercial pectin (Com Pectin) > SAFE (2 SPectin) > SAFE (1 SPectin) as indicated by all four methods of concentration detection.

In the case of MPectin, mode of detection gave the  $M_w$  values in the order, RI > 278 nm > 250 nm > 310 nm.  $M_w$  is defined by Eq. (1)

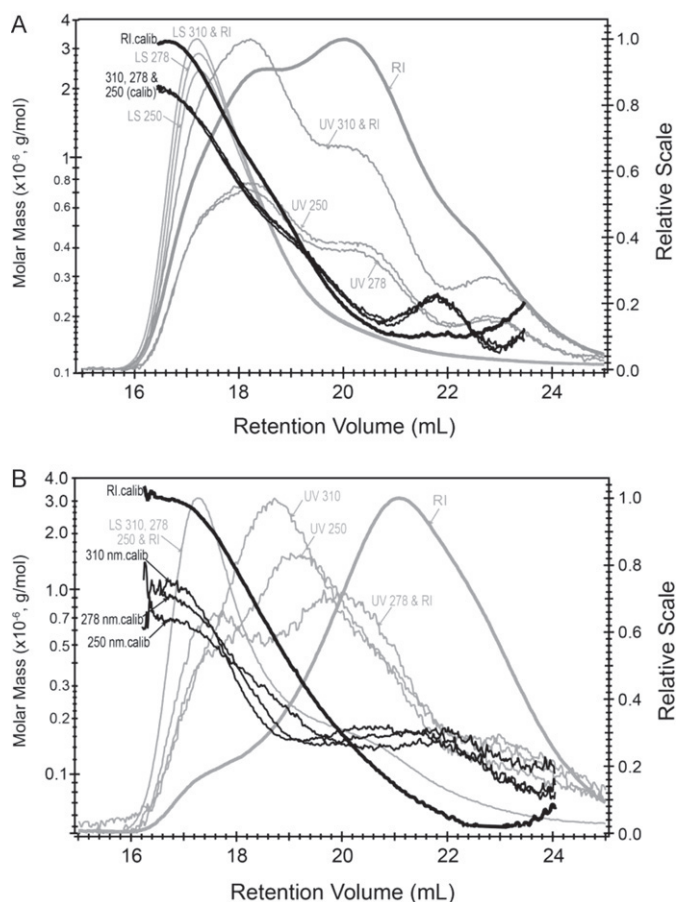
$$M_w = \sum_i C_i M_i \quad (1)$$

where  $C_i M_i$  is the fractional product of the concentration ( $C_i$ ) of each molar mass present in the distribution times the molar mass ( $M_i$ ) and  $M_w$  is the sum of all these products. In this study,  $M_w$  was calculated for each sample which was run four times by substituting separately RI, 250 nm, 278 nm, 310 nm concentration values ( $C_i$ ) into Eq. (1). The light scattered was measured during each run.

In Fig. 3 are overlay chromatograms and calibration curves detected by the four modes of concentration detection for two of the four pectin samples characterized. Fig. 3A contains the chromatogram of MPectin but also is representative of Com Pectin, and 2 SPectin. The calibration curve determines the molar mass of each molecule present whereas the area under each peak in the chromatograph determines the relative amount of each molecule present. The calibration curve and the distribution of area under the chromatogram together determine the overall molar mass, ( $M_w$ ), of the sample. The chromatograms are from the absorbance at three UV wavelengths and from the RI detector. Also present is the output from the light scattering detector at an angle of 90°. The wavelength at 278 nm rather than 286 nm was chosen because this was the wavelength that provided the best referencing signal to align the various detectors.

At low elution volumes the molar masses of the RI calibration curves are higher than those of the UV curves whereas at high elution volumes the reverse is true for all four pectins. Based on this observation, it would appear that the particles with the highest molar mass have the least amount of UV absorbing molecules associated with them. Conversely, the lower molar mass particles have the largest quantity of UV absorbing molecules associated with them. This observation appears to be inconsistent with the suggestion that the spherical particles contain protein whereas the strands are pectin. Kirby, Macdougall, and Morris (2006) were able to visualize with AFM some spherical particles with strands attached at one end. Possibly, an explanation for this inconsistency is that some strands have protein associated with them and are the ones attached to the spherical particles.

The overlay data for 1 SPectin is in 3B. Unlike the higher molar mass pectins represented by 3A, there is an appreciable separation



**Fig. 3.** HPSEC: (A) MPectin and (B) 1S Pectin. Superimposed 90° light scattering (LS), refractive index (RI), and UV (310, 278, and 250 nm) chromatograms. Molar mass calibration curves for RI and UV (310, 278, and 250 nm) were plotted as a comparison. Molar mass axis refers to calibration curves. Relative scale axis refers to chromatograms.

between the three UV calibration curves in addition to their collective separation from the RI calibration curve. Furthermore, at the high molar mass end of the UV calibration curve, i.e. low elution volumes, the relative order in molar masses of the calibration curve is 310 nm > 278 nm > 250 nm. In the intermediate molar mass range, the calibration curve order becomes 250 nm > 278 nm > 310 nm. In the low molar mass range, there is little or no separation between UV calibration curves. Comparison of the UV spectra of sugar beet with ferulic acid reveals that the spectra for sugar beet pectin exhibits a shoulder maximum at ~250 nm (spectra not shown) whereas ferulic acid exhibits a relative minimum at ~254 nm, Williams et al. (2005). All other things being equal, UV calibration curves appear to signal a reduction in protein molar mass for 1 SPectin ( $M_w$  262–272 kDa) at the high end of the molar mass distribution and an increase at the intermediate range of the distribution when compared to pectins with a higher  $M_w$ . Additionally, at the high end of the molar mass distribution there is a bigger separation between the RI calibration curve and the UV calibration curves collectively than for sugar beet pectins with larger  $M_w$  values. Based on a comparison of the UV calibration curves of Fig. 3A with those of Fig. 3B, it appears that when  $M_w$  falls below 300 kDa, the molar mass of pectin associated with protein and ferulic acid linked pectin decrease. Furthermore, the pectin associated with protein decreases in molar mass to a greater extent than ferulic acid linked pectin. Moreover, there is a greater separation between the RI calibration curve and the collective UV calibration curves indicating that pectin not bearing protein or ferulic acid has not

decreased appreciably. It should be noted that the point by point calibration curve of  $M_w$  could be slightly different from the actual  $M_w$  value in that  $dn/dc$  values could vary depending on the point by point relative ratio of pectin to protein/ferulic acid. The manufacturer supplied software does not permit calculations of  $M_w$  based on variable  $dn/dc$  values. Nonetheless, we expect that differences in calibration curves obtained from RI concentration measurements compared to UV concentration measurements are mostly due to point by point differences in the ratio of pectin to protein/ferulic acid.

As indicated above,  $M_w$  for the four sugar beet pectin samples decrease in the order MPectin > Com Pectin > 2 SPectin > 1 SPectin. With the decrease in  $M_w$ , the molar mass distribution in the chromatograms of the various samples change. In the case of MPectin, Fig. 3A, all concentration chromatograms are tri-modal in shape. Nonetheless, the lowest peak is barely visible in the RI chromatogram. Comparison of Fig. 3A with Fig. 3B reveals that the area of the first peak in the RI chromatogram decreases greatly with a concomitant increase in the second and third peak which almost completely merged. This change accounts for the higher overall molar mass of MPectin over that of the other three pectin samples.

Comparison of UV chromatograms for the various samples revealed MPectin and 1 SPectin chromatograms are similar in that they are tri modal in shape and the peaks decrease in height with increasing elution volume. Nonetheless, there is a shift from higher to lower molar mass accounting for the higher molar mass of UV detectable moieties in MPectin over those in 2 SPectin (chromatograms not shown).

Table 6 contains z-average radii of gyration ( $R_{gz}$ ) and hydrated radii ( $R_{hz}$ ).  $R_{gz}$  values ranged from 42 to 36 nm over all samples. For any particular sample, changes in radius with mode of concentration measurement were within experimental error. Changes with sample molar mass were either negligible or within experimental error.  $R_{hz}$  values obtained with the RI detector were appreciably larger than those obtained with the UV detector. Table 7 contains the ratio of  $R_{gz}/R_{hz}$  ( $\rho$ ) which is a measure of macromolecular compactness (Burchard, 1996). Values of  $\rho$  are, for the most part, larger for particles measured by UV than for particles measured by RI. Thus particles measured by RI are not only larger in molar mass but more compact than UV absorbing particles.

As expected MPectin has a larger  $\eta_w$  than the other three pectins. Rather unexpectedly, given its lower  $M_w$ ,  $\eta_w$  of 1 SPectin is about the same as COM Pectin and 2 SPectin (Table 5).

Previously we showed with AFM, that when pectin which underwent microwave assisted extraction (MPectin) was dissolved in pure water at a concentration of 12.5  $\mu\text{g/mL}$  it formed a network structure which could be dissociated into its component parts when dissolved at one half the concentration (6.25  $\mu\text{g/mL}$ ) (Fishman et al., 2008). Dissociation of the network structure revealed that the network was comprised of individual molecules with a variety of shapes. Pectin networks were found to be comprised of spherical molecules some of which were connected through linear strands whereas others were only connected to one or more strands. Other pectin molecules found to be present were stranded molecules not associated with spherical molecules. The shapes of these included rods, kinked rods, segmented rods, and closed loop chains. HPSEC analysis with online light scattering, viscosity and RI detection revealed three regimes of separation when the chromatogram of MPectin was integrated by parts. One regime contained compact, possibly spherical particles, with an  $M_w$  of 3700 kDa. The second or transition regime had an  $M_w$  of 1300 kDa. The third regime had an  $M_w$  of 390 kDa and contained particles which were spatially equivalent to an expanded coil. A material balance revealed the first regime contained about 7.8%, the second about 59.5% and the third about 34.0% of total material. Presumably, the second

regime contained materials from both the first and third regimes. In other AFM studies, Kirby et al. (2006) suggested that the spherical molecules were protein and the molecular strands were pectin, in that the number of stranded molecules far outweighed the number of spherical molecules.

Williams et al. (2005) found that sugar beet pectin had UV absorbance peaks at  $\sim 200$ ,  $\sim 250$ ,  $\sim 286$  and  $\sim 310$  nm whereas ferulic acid also had significant absorption at  $\sim 231$ ,  $\sim 294$  and  $\sim 319$  nm (Sebastian et al., 2009). Extensin (associated with sugar beet pectin) is hydroxyproline-rich with lesser amounts of tyrosine and phenylalanine but no detectable tryptophan. The amino acid composition of extensin is consistent with the UV absorbance observed by Williams et al. (2005). Based on the amino acid composition of extensin, one would expect it to have significant absorbance contributions over the range 260–290 nm and below 230 nm.

Nunez et al. (2009) employing MALDI-TOF/TOF-MS identified extensin as the protein associated with sugar beet pectin. Extensin is hydroxyproline-rich with lesser amounts of tyrosine and phenylalanine but no detectable tryptophan. The amino acid composition of extensin is consistent with the absorbance observed by Williams et al. (2005). Based on the amino acid composition of this protein, one would expect it to have significant absorbance contributions over the range 260–290 nm and below 230 nm.

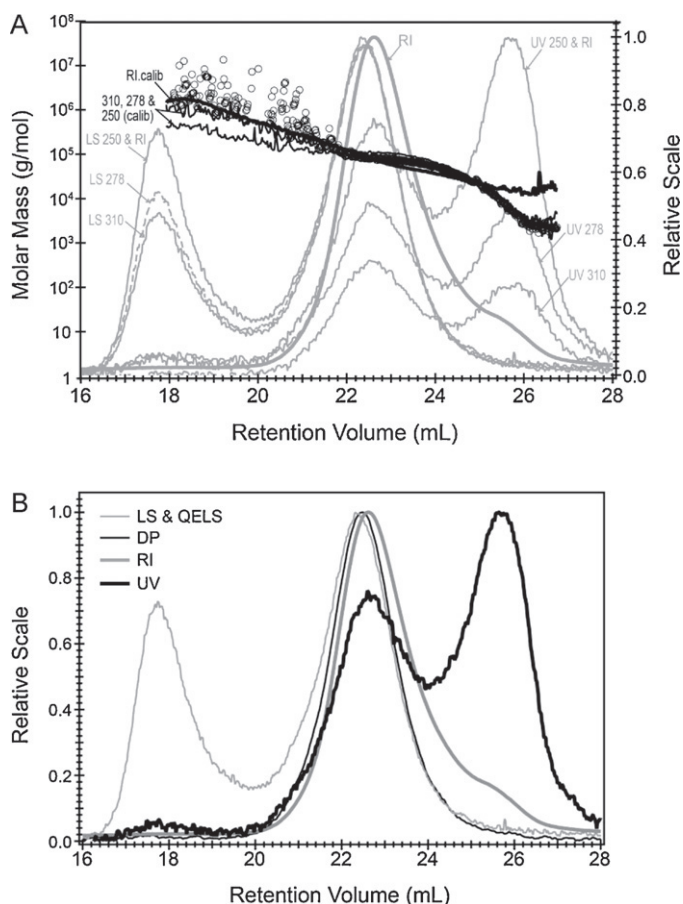
### 3.3. Characterization of the molecular properties of ASP I

Fig. 4A contains overlay chromatograms and calibration curves detected by the four modes of concentration detection for 1 ASP I. The trimodal chromatogram shows signals from all four concentration detectors. The 310 nm wavelength gave the lowest signal relative to the other concentration detectors for 1 ASP I whereas it gave the largest signal relative to the other concentration detectors for pectin. The existence of a 310 nm peak in the 1 ASP I chromatogram, albeit smaller than found in pectin chromatograms, signifies the presence of some ferulic acid in 1 ASP I. This in turn indicates the presence of some RG-I fragment of pectin in 1 ASP I (Morris et al., 2010). The presence of protein in addition to some pectin is also consistent with the presence of UV absorption at 278 nm and 250 nm. Fig. 4B contains chromatograms from four detectors, the LS, RI, 250 nm UV and the viscosity detector (DP). Only the middle peak shows a viscosity signal whereas the first and third peaks gave no viscosity signal. Thus, most of the high molar mass polysaccharide is contained in the middle peak. The first peak shows a large LS peak and small RI and UV peak. This behavior may indicate that the first peak consists of a small amount of loosely re-aggregated pectin fragments. The last peak probably is due to non-aggregated low molecular weight fragments. In most cases, values of  $M_w$ ,  $\eta_w$ ,  $R_{gz}$  and  $R_{hz}$  for 2 ASP I (Tables 5–7) are comparable to those found for 1 ASP I.  $M_w$  values tended to decrease in the order  $RI \geq 250 \text{ nm} \geq 278 \text{ nm} \geq 310 \text{ nm}$ . These results also suggest a fraction which contains some protein. Furthermore closely aligned and fairly straight calibration curves in Fig. 4A from the four concentration detectors also are consistent with the ASP I fraction containing protein. Nevertheless, the appreciably lower value of  $\eta_w$  for ASP I compared to that of pectin is an indication of the relatively lower molar mass of ASP I (Table 5).

### 3.4. Characterization of the molecular properties of ASP II

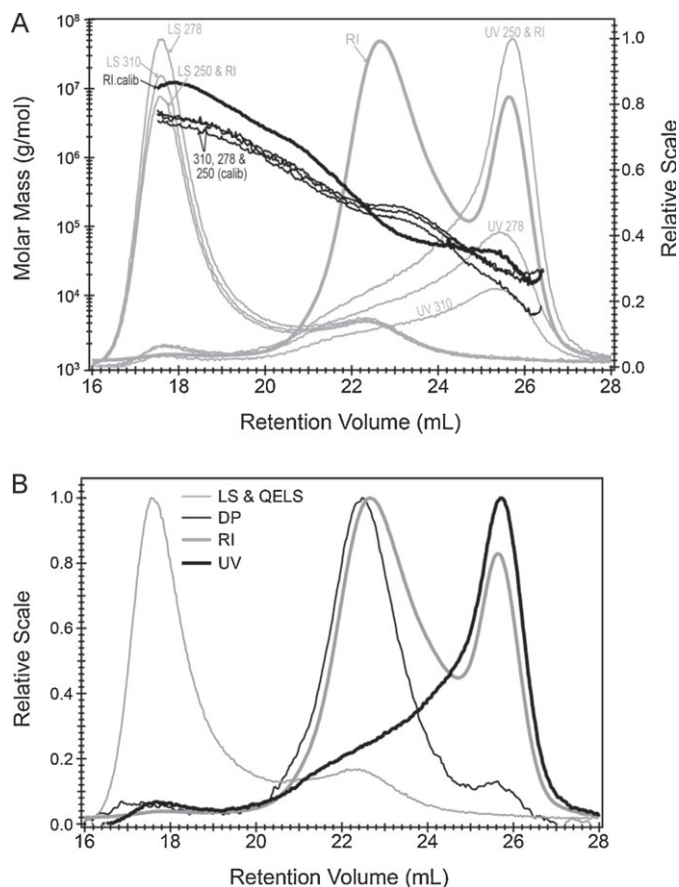
The  $M_w$  of 1 and 2 ASP II fractions was appreciably higher than that of ASP I when subjected to SAFE (Table 5). When subjected to MAFE,  $M_w$  of ASP I and ASP II were comparable, Fishman et al. (2009). Unlike 1 ASP I and 2 ASP I, the  $M_w$  of 1 ASP II is appreciably smaller than 2 ASP II when subjected to SAFE (Table 5). In Figs. 5A and 6A are overlay chromatograms and calibration curves





**Fig. 4.** HPSEC: 1 ASP I. (A) Superimposed 90° light scattering (LS), refractive index (RI) and UV (310, 278, and 250 nm) chromatograms. Superimposed molar mass calibration curves for RI, UV (310, 278, and 250 nm). (B) Superimposed 90° light scattering (LS), refractive index (RI), viscosity (DP), and UV (250 nm) chromatograms. Molar mass axis refers to calibration curves. Relative scale axis refers to chromatograms.

detected by the four modes of concentration detection for 1 ASP II and 2 ASP II respectively. Also included in these figures are the light scattering chromatograms detected for each of the concentration detectors. The chromatograms in Figs. 5A and 6A are trimodal. The small peaks in Fig. 5A between 17 and 18 mL are probably due to a small amount of non-fragmented high molar mass pectin associated protein. This conclusion is supported by an RI calibration curve which is slightly higher than the three UV calibration curves (Fig. 5A) and a small but measurable amount of viscosity, both of which are at the high molar mass end in Fig. 5B. In 1 ASP II there is a large middle DP peak and a small LS peak (Fig. 6B) indicating that it is where most of the high molar mass polysaccharide is located. Sample 2 ASP II has the highest  $M_w$  (Table 5) and amount of soluble protein (Table 4) of ASP samples. Also, the UV chromatographic concentration peaks at the high molar mass end are much larger for 2 ASP II (Fig. 6A) than for the high molar mass end for 1 ASP I. Furthermore, the viscosity curve for 2 ASP II (Fig. 6B) in the middle molar mass region has much larger area than for the other three ASP samples. Moreover, unlike all of the other ASP samples, the 2 ASP II sample has measurable viscosity in the lowest molar mass peak. Based on  $M_w$  and  $\eta_w$  values in Table 5, we conclude that the 2 ASP II contains RG-I and HG fragments of pectin. Furthermore, monosaccharide analysis data in Table 3 also is consistent with 2 ASP II containing RG-I and HG fragments. Morris et al. (2010) have isolated HG and RG-I from sugar beet pectin. They found that RG-I fragments had an 188 kDa  $M_w$  value and an 0.36 dL/g  $\eta_w$  value



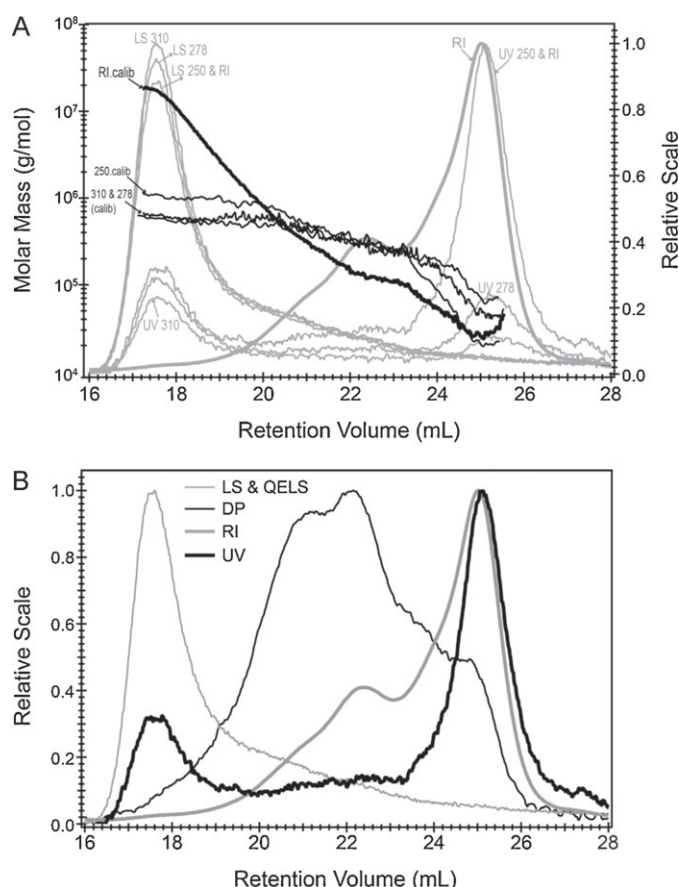
**Fig. 5.** HPSEC: 1 ASP II. (A) Superimposed 90° light scattering (LS), refractive index (RI), and UV (310, 278, and 250 nm) chromatograms. Superimposed molar mass calibration curves for RI, UV (310, 278, and 250 nm). (B) Superimposed 90° light scattering (LS), refractive index (RI), viscosity (DP), and UV (250 nm) chromatograms. Molar mass axis refers to calibration curves. Relative scale axis refers to chromatograms.

whereas HG values were 20 kDa and 0.77 dL/g respectively. For 2 ASP II we found an average  $M_w$  value of 364 kDa  $M_w$  and an average  $\eta_w$  value of 0.30 dL. Nonetheless, sample 2 ASP II also contains a much larger percentage of galacturonic acid and protein than the other three ASP samples (Tables 2–4) which indicates the presence of HG. The data in Table 2 indicates the absence of ester or acetyl groups in all of the ASP fractions due to alkaline extraction conditions. Therefore the pectin present in these fractions would appear to be there without ester or acetyl groups.

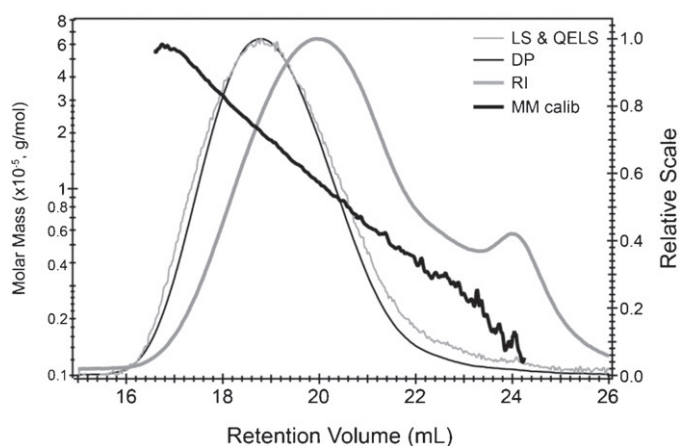
### 3.5. Characterization of the molecular properties of CMC

Tables 3 and 4 indicate that there may have been trace amounts of other carbohydrates and proteins in the CMC fractions. Nonetheless these were insufficient to obtain “good” chromatographic data with the UV detectors unlike the RI detector. In Fig. 7 are chromatograms with superimposed calibration curves for 2 CMC (S). These chromatograms are representative for both 1- and 2 CMC (S).  $M_w$  was 263 kDa and 127 kDa,  $\eta_w$  was 8.0 and 3.6 dL/g,  $\rho$  was 1.1 and 1.3, respectively for these CMCs (Tables 5 and 7). A  $\rho$  value of 0.78 has been associated with compact spheres whereas a value  $>2$  has been associated with rod-like polymers (Burchard, 1996). In previous AFM work, Fishman et al. (2011) found that CMC from the cellulose in sugar beet pulp was a mixture of rods and hard spheres which would explain the  $\rho$  values found in this work.





**Fig. 6.** HPSEC: 2 ASP II. (A) Superimposed 90° light scattering (LS), refractive index (RI), and UV (310, 278, and 250 nm) chromatograms. Superimposed molar mass calibration curves for RI and UV (310, 278, and 250 nm). (B) Superimposed 90° light scattering (LS), refractive index (RI), viscosity (DP), and UV (250 nm) chromatograms. Molar mass axis refers to calibration curves. Relative scale axis refers to chromatograms.



**Fig. 7.** HPSEC: 2 CMC (S). Molar mass calibration curves are superimposed on 90° light scattering (LS), refractive index (RI), and viscosity (DP) chromatograms. Molar mass axis refers to calibration curves. Relative scale axis refers to chromatograms.

#### 4. Conclusion

MAFE and SAFE pectin require significantly less extraction heating time than commercial pectin. Presently, it appears that MAFE produces pectin with higher molar mass and viscosity than either commercial pectin or SAFE pectin. The advantage of SAFE pectin over MAFE pectin is that it would be less costly and easier

equipment wise for manufacturers to convert their present process for producing pectin to the MAFE process. Furthermore, the SAFE process could be improved with additional engineering of the process.

Compositional and multi-detector HPSEC analysis of SBP polysaccharides revealed that alkaline soluble fractions contained pectin with a different composition compared to the acid soluble polysaccharides. The ratio of galacturonate to crude protein was about 5:1 and about 8% of the crude protein was not associated with galacturonate. Peak areas together with  $M_w$  calibration curves using UV and the RI concentration detectors indicated that the molar masses of UV absorbing molecules was skewed to the lower end of the molar mass distribution. Nevertheless, based on AFM measurements (Fishman et al., 2008; Kirby et al., 2006), it is possible that some strands of pectin associated with protein are attached to high molar mass spherical pectin molecules. Using pectins with four different values of  $M_w$  allowed us to study the effect of molar mass distribution on the global structure of sugar beet pectin. For  $M_w$  below 300 kDa, the molar mass of pectin associated with protein and ferulic acid linked pectin decrease, protein associated pectin molar mass decreases to a greater extent than ferulic acid linked protein. Furthermore, pectin not bearing protein or ferulic acid has not decreased appreciably.

Characterization of ASP I indicated that there was a rhamnogalacturonan I pectin and associated protein in this fraction. Characterization of ASP II indicated that  $M_w$  of ASP II was appreciably higher than ASP I which was probably due to a larger amount of homogalacturonan pectin and associated protein in ASP II than ASP I.

The value of  $\rho$  in the CMC fraction possibly indicated the presence of rod-like and less extended molecules. This may indicate the presence of ASP or another neutral polysaccharide in the CMC fraction. Monosaccharide analysis also indicated the presence of a small amount of a neutral polysaccharide in this fraction. Crude protein analysis revealed that there was little if any protein in the CMC fraction.

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